

Original Article

Echinacoside protects adenine-induced uremic rats from sciatic nerve damage by up-regulating α -Klotho

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Abstract

Objectives: To investigate the therapeutic effect of Echinacoside on uremia-induced sciatic nerve injury and explore the specific molecular mechanism and role of α -Klotho. **Methods:** SD rats were given continuous gavage of adenine to prepare a uremia-induced sciatic nerve injury model. The model was given either Echinacoside or α -Klotho by gavage. Histopathological changes of kidney and sciatic nerve were detected by H&E staining. The changes of creatinine, urea nitrogen, and urine protein were detected by biochemical detection. The changes of IL-1 β and IL-18 were detected by ELISA. Nerve activity-related indicators were detected by biochemical detection. Changes in related mRNA and protein expression were detected by qPCR and western blot. **Results:** Creatinine, urea nitrogen, urine protein, and malondialdehyde (MDA) in the model group were significantly increased and inhibited by Echinacoside and α -Klotho treatment with Echinacoside dose-dependence. Meanwhile, the activities of ATP concentration, potassium adenosine triphosphate (Na⁺, K⁺ ATPase), succinate dehydrogenase (SDH), glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD) showed opposite trends. **Conclusions:** Echinacoside can significantly relieve uremia-induced sciatic nerve injury in rats. Its specific molecular mechanism is related to the inhibition of the classical cellular pyroptosis pathway, which is likely achieved by promoting α -Klotho expression.

Keywords: α -Klotho, Echinacoside, Uremia-Induced Sciatic Nerve Injury

Introduction

Peripheral neuropathy (PN) is a disorder that affects the cell body, axon, or myelin of motor or peripheral sensory neurons¹, which is characterized by persistent and recurrent pain. The overall prevalence is about 2.4%. However, this data dramatically increases at a given age, with about 8% of

the population over 55². PN inducing factors can be divided into nutritional deficiency, toxic neuropathy, and clinical prognosis. In addition, 60-100% of patients undergoing dialysis due to chronic kidney disease (CKD) are found peripheral neuropathy³. Uremic neuropathy (UN) occurs when renal insufficiency is impaired by filtration, leading to the accumulation of organic waste. This accumulation is particularly significant when glomerular filtration rate (GFR) decreases in patients with end-stage renal disease (ESRD)^{4,5}. Neuropsychiatric symptoms of the patients are associated with various factors, such as uremic toxin storage, metabolic acidosis, and electrolyte disorders^{6,7}. From the preclinical point of view, the peripheral nerve structure is complex and can only be partially simulated *in vitro*. Therefore, the study of its repair and regeneration still needs to be carried out on animal models. Sciatic nerve injury model is the most common⁸.

Renal transplantation and dialysis are the main treatment methods in patients with end-stage nephropathy, with high costs. Among them, kidney transplantation is less used

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because of insufficient donors. Dialysis is associated with central nervous system (CNS) diseases, such as dialysis imbalance syndrome, dialysis dementia, and progressive mental retardation^{9,10}. Because of the deficiency of the above two treatment methods, it is critical to find other more efficient drugs to treat uremic neuropathy. On the other hand, natural products have a broad prospect in uremic neuropathy due to their good safety profile and low toxicity. Echinacoside is a natural compound isolated from *Echinacea angustifolia* DC¹¹. It was been shown to possess numerous pharmacologically beneficial activities for human health, especially the cardiovascular and anti-osteoporotic effect, neuroprotective, anti-inflammatory and antioxidant activities¹². Echinacoside can improve the antioxidant, anti-fatigue, and anti-stress ability of vascular dementia rats or subacute aging mice *in vivo* experiments and play an antioxidant role indirectly by the induction or activation of endogenous major antioxidant enzymes and inactivation of oxidase promoters¹³. This suggests that Echinacoside may relieve uremic neuropathy. However, there are still no relevant reports, and the specific mechanism of this activity still remains unclear.

α -Klotho is an anti-aging protein enriched in the kidneys, reported to have anti-oxidation, anti-aging, anti-apoptosis, vascular protection, and other effects¹⁴⁻¹⁶. In addition, among the various nephrotic models, including CKD, the expression of α -Klotho was significantly down-regulated¹⁷. Supplementation of exogenous α -Klotho could help prevent, delay, and alleviate kidney injury^{18,19}. And these studies suggest that α -Klotho plays an important role in kidney damage and protection. However, its role in sciatic nerve injury caused by uremia has not been confirmed. In order to explore the role of Echinacoside in relieving sciatic nerve injury caused by uremia, we established a uremic rat model. Furthermore, the effects of Echinacoside on neurophysiological and histopathological responses were studied in rats with sciatic nerve injury. The role of α -Klotho in this process and related regulatory mechanisms was further explored.

Material and Methods

Animals

Sprague Dawley (SD) rats were purchased from Shanghai Laboratory Animal Center (Shanghai, China). All rats were placed in a temperature-controlled room ($25\pm 1^\circ\text{C}$) and had free access to food and drink. All animal experiments were conducted in strict accordance with the International Ethical Guidelines and Guidelines for the Care and Use of Experimental Animals from the National Institutes of Health. This study was approved by the Ethics Committee of Animal Experiments of the Jing' an District Centre Hospital of Shanghai, Fudan University.

Experimental Group

After a week of adaptive feeding, 36 adult male SD rats (6 weeks old, weighing 200-220 g) were randomly

divided into 6 groups: normal group (NC), Model group (Model), Model+Echinacoside (10 mg/kg/day) group (L), Model+Echinacoside (20 mg/kg/day) group (M), Model+Echinacoside (40 mg/kg/day) group (H), Model+ α -Klotho proteome (injection, 0.02 mg/kg/day) (Klotho).

Adenine-induced uremia rat model was obtained by continuous oral administration of adenine (2.5%, 250 mg/kg/d) once every two days for 14 days. When modeling, the rats in the Echinacoside group was given the corresponding concentration of Echinacoside intragastric treatment once every two days until the end of modeling. Subcutaneous injection of recombinant α -Klotho protein (0.02 mg/kg/d) were administered in the rats in the Klotho group once a day for 3 days at the first time of adenine gavage. Simultaneously, the rats in the normal group and the uremic group were given the equivalent volume of normal saline. Blood and urine samples were collected two weeks later. The rats were sacrificed to obtain the kidneys and sciatic nerve tissues.

H&E Staining

Histological analysis on renal and sciatic nerves: The above-obtained tissues were fixed with 10% neutral buffered paraformaldehyde, dehydrated in graded concentrations of ethanol, immersed in xylene, and embedded in paraffin. Sections of 4 μm thickness were cut, stained with hematoxylin and eosin, and evaluated under optical microscope. Histopathological changes were observed.

Creatinine, urea nitrogen, and urine protein assay

Serum creatinine, urine total protein, and urea nitrogen concentrations were determined by colorimetric method according to the specification of the commercial kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Analysis on the activity of nerve cells

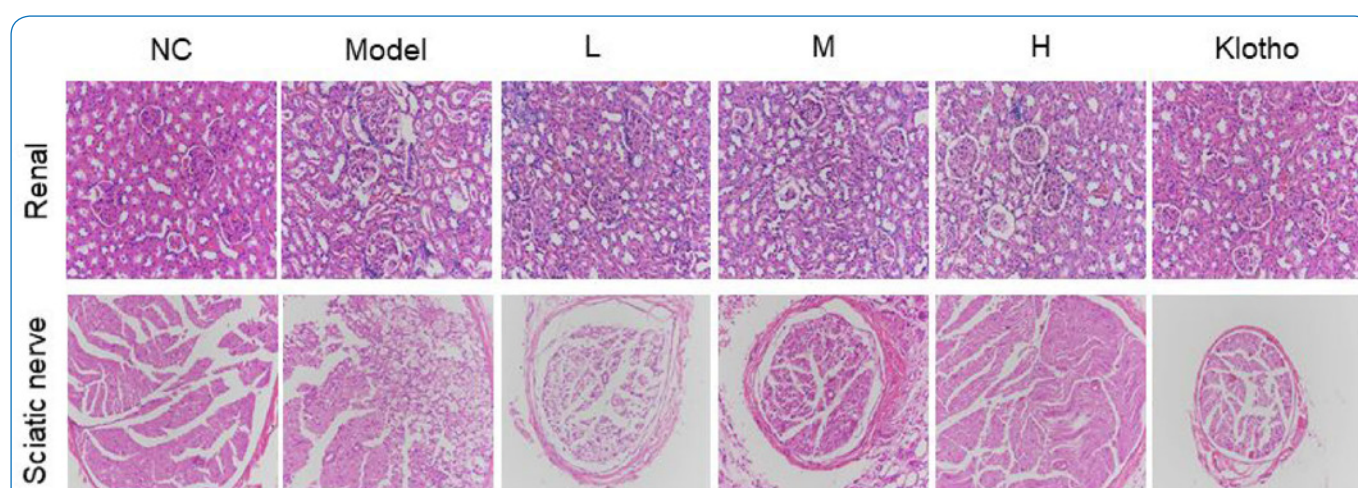
Following the manufacturer instructions, the total protein and malondialdehyde (MDA) concentration and ATP content, sodium, potassium, adenosine triphosphatase (Na^+ , K^+ -ATPase) and succinate dehydrogenase (SDH), glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD) activity were biochemically analyzed with commercial kits (Nanjing City Biological Engineering Research Institute). Total protein concentration was expressed in $\mu\text{g}/\text{ml}$. Malondialdehyde (MDA) concentration was expressed in $\text{mM}/\mu\text{g}$ of protein. ATPase, Na^+ - K^+ ATPase, SDH, and SOD activities were expressed in U/mg of protein. GSH-Px activity was expressed in U/g of protein.

ELISA

IL-1 β and IL-18 in blood samples were detected by ELISA. The blood samples were centrifuged at 18000 \times g, 4 $^\circ\text{C}$ for 15 minutes. Serum samples were obtained. Then, according to the instructions, IL-1 β and IL-18 ELISA detection kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and

Table 1. Primers used in q-RT PCR Assays.

Gene name	Sequence (5'-3')	Description
Klotho	TGTCTATCTGTGGGATGTGC	Forward
Klotho	CGAAGTAAGGTTATCTGAGGC	Reverse
NF- κ B p65	GACCTGCCTGAAGCTACTC	Forward
NF- κ B p65	TGGTTTCCATACCGTGCC	Reverse
NLRP3	CCAGGGCTCTGTTCATTG	Forward
NLRP3	ACCTTCACGTCTCGGTTTC	Reverse
GAPDH	GGAGTCTACTGGCGTCTTCAC	Forward
GAPDH	ATGAGCCCTTCCACGATGC	Reverse

**Figure 1.** H&E detects Echinacoside and α -Klotho of adenine-induced renal and sciatic nerve histopathological changes in rats with uremia sciatic nerve injury model ($\times 200$). NC: normal control group; L: Model + Echinacoside (10 mg/kg/day); M: Model + Echinacoside (20 mg/kg/day); H: Model + Echinacoside (40 mg/kg/day).

automatic biochemistry analyzer (HITACHI 7170S, Japan) was used to determine IL-1 β and IL-18 concentrations.

qPCR

Total RNA was extracted from sciatic nerve tissue with Trizol reagent. RNA integrity was examined on 2% agarose gel, and RNA content was determined with a spectrophotometer (Thermo, Waltham, MA). cDNA was reversed from transcription of RNA (2 μ g per sample) using cDNA Synthesis reagents.

Primers used for real-time PCR (Table 1) were designed by Primer 5.0 and synthesized by JRDun Biotech (Shanghai, China). PCR reaction was performed with SYBR Green reagents (Thermo, USA) on quantitative real-time PCR instrument (ABI-7300, USA). The relative expression mRNA was calculated by $2^{-\Delta\Delta Ct}$ (20), with GAPDH as the sample control.

Western Blot

The total protein of sciatic nerve tissue was extracted. First, the nucleus protein was extracted by nucleus protein extraction kit (Beyotime Bio, China). The content was detected by BCA protein detection kit (Thermo, Waltham, MA) according to the instructions. Then, the protein (30 μ g/samples) with 10% SDS-PAGE was isolated and transferred to the PVDF membrane, sealed in 5% skim milk under 25°C for 1 h, and incubated with primary NF-B P65 (1:2000, Abcam Biotech, Cambridge, MA, USA), Caspase1 (1:1000, Abcam), Pro-caspase1 (1:1000, Cusabiao, Wuhan, China), GSDMD-N (1:1000, Abcam), Klotho (1:1000, Abcam), NLRP3 (1:1000, Abcam), GAPDH (1:2500, Abcam) and H3 (1:2000, Abcam). And then, it was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Beyotime, Shanghai, China) for 1 h. After that, protein bands were detected with a ECL detection kit (Beyotime Biothech, Shanghai, China), GAPDH or H3 was taken as control.

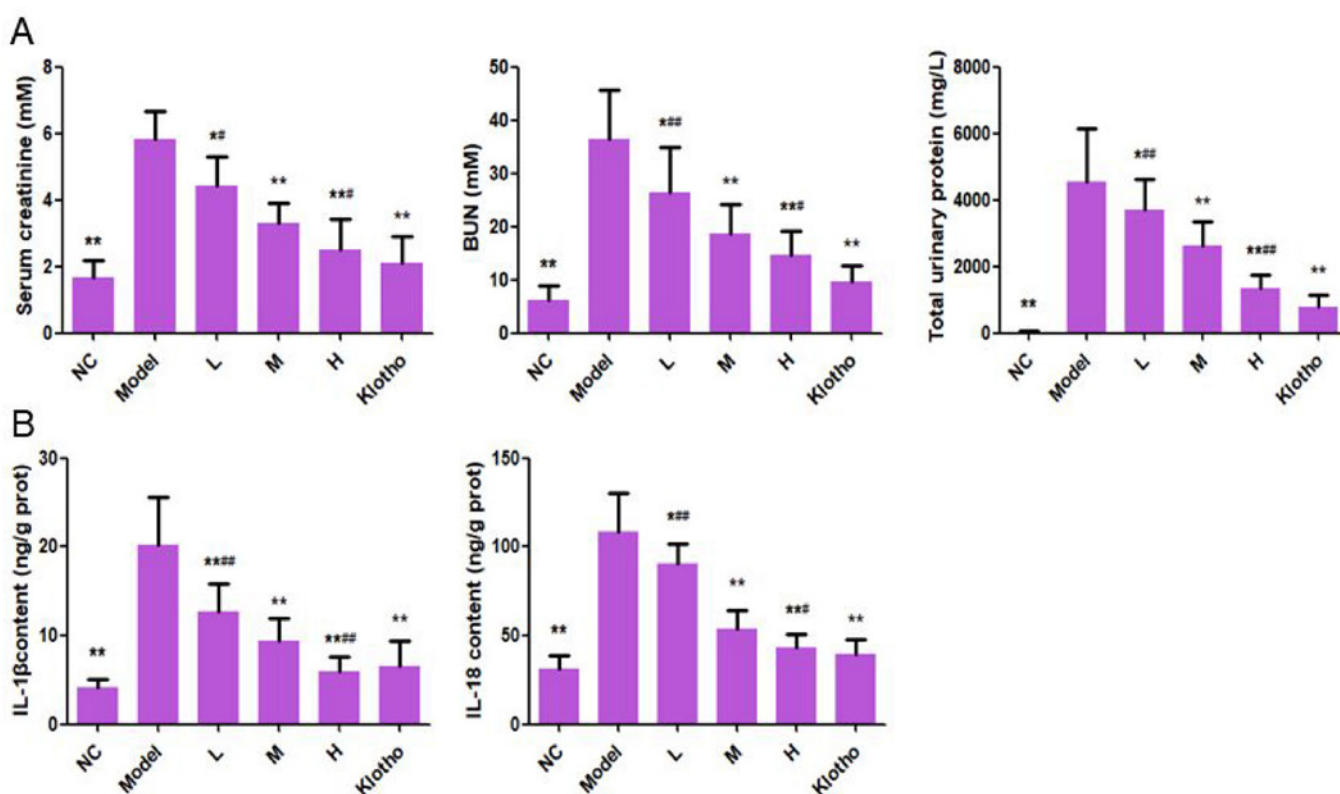


Figure 2. Echinacoside and α -Klotho improve renal function in uremia-induced sciatic nerve injury rats. (A) The increased IL-1 β and IL-18 contents of sciatic nerve injury rats was decreased by Echinacoside and α -Klotho; (B) NC: normal control group; L: Model + Echinacoside (10 mg/kg/day); M: Model + Echinacoside (20 mg/kg/day); H: Model + Echinacoside (40 mg/kg/day); * p <0.05; ** p <0.01 VS model group, # p <0.05; ## p <0.01; L and H groups VS M group.

Statistical Analysis

All data were expressed as mean \pm standard error of the mean (SEM) and the one-way analysis of variance (ANOVA) was performed using SPSS Statistics 20.0 statistical software. P <0.05 was considered to be statistically significant.

Results

Histopathological Analysis

Hematoxylin-eosin staining (H&E) analyses in renal tissues and sciatic nerve tissues were performed to observe histopathologic changes. The adenine-induced uremic model of rats was successfully established at the histological level (Figure 1). The results showed that the normal group had normal renal structure and complete glomeruli surrounded by renal vesicles and tubules. The morphological changes of interstitial edema and neutrophil aggregation in the model group were obvious; renal damage and inflammatory reaction were obvious compared with NC group. Moreover, in Echinacoside and α -Klotho groups, the renal injury was significantly alleviated with Echinacoside dose-dependent (Figure 1).

The results of H&E staining of sciatic nerve showed that the sciatic nerve fibers in NC group were arranged neatly

and orderly. However, the nerve fibers were thin, and sciatic nerve injury was observed in the model group. The sciatic nerve injury was significantly alleviated in all Echinacoside and α -Klotho groups, with Echinacoside in a dose-dependent manner (Figure 1).

Echinacoside and α -Klotho improve renal function in rats with uremia

To further analyze the effects of Echinacoside and α -Klotho on renal injury of uremia in rats, creatinine in blood/urine, urea nitrogen, and urinary protein content were tested. The contents of creatinine, urea nitrogen, and urine protein in the model group were significantly higher than those in the normal group, which could be improved by Echinacoside and α -Klotho treatment (Figure 2A). Moreover, the dosage of Echinacoside reduced creatinine, urea nitrogen, and urinary protein in a dose-dependent manner (Figure 2A). Meanwhile, according to the ELISA results of the IL-1 β and IL-18 of inflammatory factors in the blood of each group, IL-1 β and IL-18 were increased in the content model group, Echinacoside and α -Klotho can reduce the content of IL-1 β and IL-18 in rats with renal injury (Figure 2B).

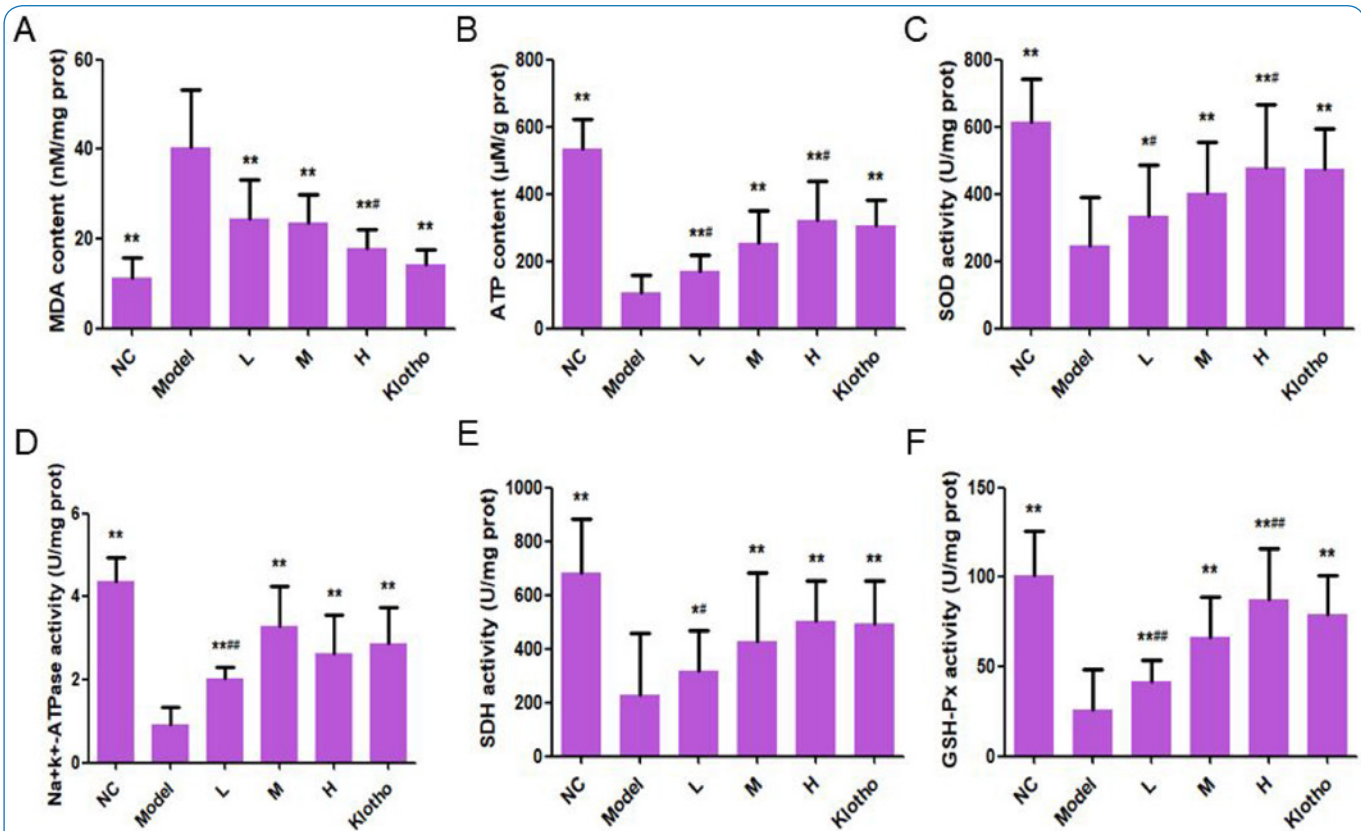


Figure 3. Echinacoside and α -Klotho improve sciatic nerve activity in sciatic nerve injury rats. (A) MDA content, (B) ATP content, (C) SOD activity, (D) Na⁺-K⁺ ATPase activity, (E) SDH, (F) GSH-Px activity. NC: normal control group; L: Model + Echinacoside (10 mg/kg/day); M: Model + Echinacoside (20 mg/kg/day); H: Model + Echinacoside (40 mg/kg/day); *p<0.05; **p<0.01 VS model group, #p<0.05; ##p<0.01; L and H groups VS M group.

Echinacoside and α -Klotho improve sciatic nerve cell activity injury in rats with uremia

To evaluate the repair effects of Echinacoside and α -Klotho on uremia-induced sciatic nerve injury in rats, we further examined the content of sciatic nerve MDA, ATP, and the activity of SOD, Na⁺-K⁺ ATPase, SDH, and GSH-Px. Experimental results showed that the MDA content in the model group was higher than that in the normal control group but decreased after Echinacoside and α -Klotho treatment. Meanwhile, with the increase of Echinacoside treatment concentration, the MDA content was further reduced. In addition, ATP content and activities of SOD, Na⁺-K⁺ ATPase, SDH, and GSH-Px in sciatic nerve tissues showed an opposite trend to MDA content (Figure 3).

α -Klotho, NLRP3 and NF- κ B p65 mRNA expression changes

To analyze the molecular mechanism of Echinacoside and α -Klotho for repair of uremia-induced sciatic nerve injury in rats, changes in α -Klotho, NLRP3, and NF- κ B p65 mRNA expression were detected. The results showed that the expression of α -Klotho was down-regulated in the model group, but the α -Klotho mRNA level was up-regulated in

Echinacoside treatment. There was no significant difference between middle and low doses, and α -Klotho treatment did not affect the level of α -Klotho mRNA in the model. Moreover, NLRP3 and NF- κ B p65 mRNA expression were up-regulated in the model group. NLRP3 and NF- κ B p65 mRNA levels were down-regulated by Echinacoside and α -Klotho, with Echinacoside in a dose-dependent manner (Figure 4).

Effects of Echinacoside and α -Klotho on the expression of sciatic nerve tissue protein in rats with uremia

We further examined the changes in the expression of NF- κ B p65 (nuclei and plasma), Caspase1, Pro-Caspase1, GSDMD-N, Klotho, and NLRP3 proteins. As shown in Figure 5, the expression of Caspase1, Pro-Caspase1, and GSDMD-N was up-regulated in the model. Echinacoside and α -Klotho significantly improved this phenomenon. In addition to the low concentration of Echinacoside, the expression of Caspase 1 was not significantly different, and the Echinacoside treatment showed a significant dose-effect (Figure 5). On the other hand, the protein expression levels of NF- κ B p65 (plasma) and α -Klotho were down-regulated in the model. Echinacoside and α -Klotho significantly up-regulated the

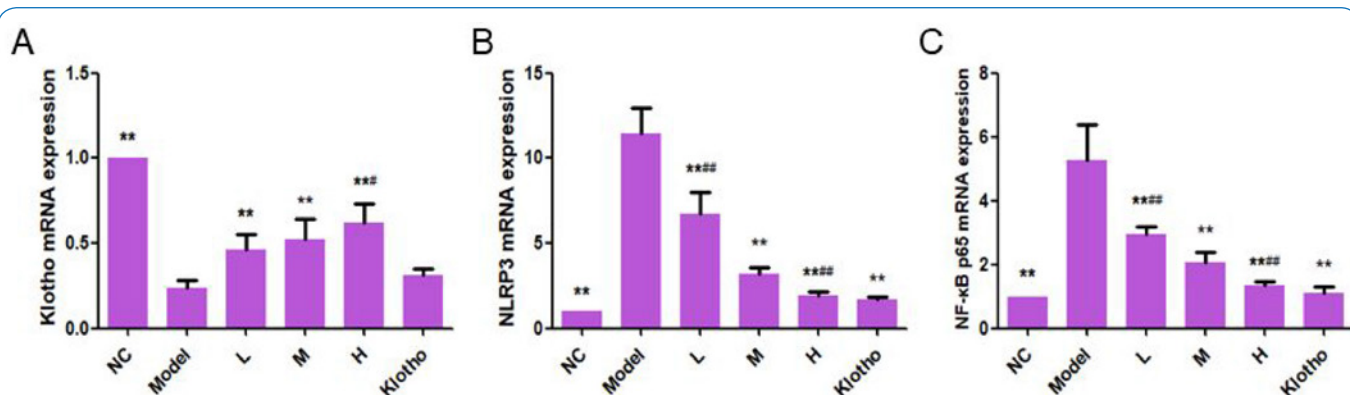


Figure 4. The expression of α -Klotho mRNA in sciatic nerve injury rats is inhibited (A), and the expression of NLRP3 (B) and NF- κ B p65mRNA (C) is up-regulated. Echinacoside reverses this phenomenon. α -Klotho inhibits NLRP3 and NF- κ B p65mRNA expression in rats. NC: normal control group; L: Model + Echinacoside (10 mg/kg/day); M: Model + Echinacoside (20 mg/kg/day); H: Model + Echinacoside (40 mg/kg/day); * $p < 0.05$; ** $p < 0.01$ VS model group, # $p < 0.05$; ## $p < 0.01$; L and H groups VS M group.

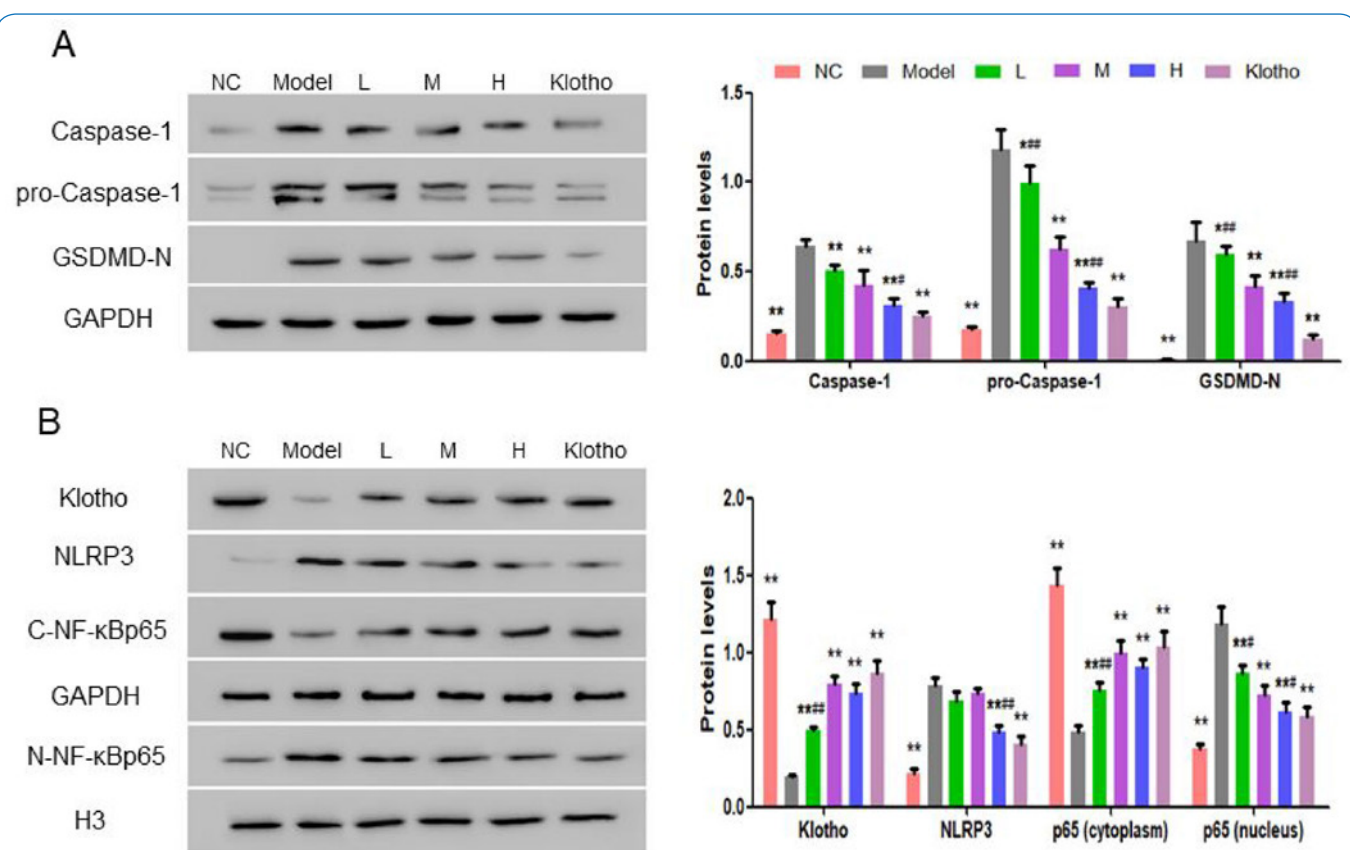


Figure 5. The effects of Echinacoside and α -Klotho on the expression of α -Klotho and cell pyrolytic related proteins. (A) Changes in protein expression of Caspase1, Pro-Caspase1, GSDMD-N. (B) Changes in protein expression of Klotho, NLRP3, NF- κ B p65 (plasma), and NF- κ B p65 (nuclear). NC: normal control group; L: Model + Echinacoside (10 mg/kg/day); M: Model + Echinacoside (20 mg/kg/day); H: Model + Echinacoside (40 mg/kg/day); * $p < 0.05$; ** $p < 0.01$ VS model group, # $p < 0.05$; ## $p < 0.01$; L and H groups VS M group.

protein expression levels of NF- κ B p65 (plasma) and α -Klotho. In addition, NLRP3 protein expression was enhanced in the model group. Echinacoside and α -Klotho significantly

decreased NLRP3 protein levels. NF- κ B p65 (nucleus) showed the same trend as NLRP3 in protein expression, but Echinacoside showed significant dose effects (Figure 5).

Discussion

Peripheral neuropathy is one of the most common neurological diseases. Although nerve damage usually does not threaten the life of patients, it imposes a heavy economic and social burden in case of long-term disability²¹. Currently, scholars in this field are committed to the study of effective treatment of peripheral nerve injury to promote tissue regeneration and functional recovery and ultimately apply it to patients to improve clinical efficacy²². Because of the complexity of the nerve organs, *in vitro* studies of nerve regeneration and repair are limited. Preclinical studies were conducted mainly on animal models. However, sciatic nerve injury has become the most common experimental paradigm for preclinical study of peripheral nerve regeneration because of its large structure, high incidence, and simple modeling method^{23,24}. In addition, there is a close correlation between peripheral neuropathy and CKD³. In this study, we developed a uremia-induced sciatic nerve injury model, and we examined the therapeutic effects of Echinacoside and α -Klotho treatment. Experimental results showed that Echinacoside and α -Klotho could effectively improve urinary symptoms. Renal function indicator creatinine, urea nitrogen, and urine proteins were significantly increased in the model and enhanced by Echinacoside and α -Klotho (Figure 1 and Figure 2). Moreover, Echinacoside and α -Klotho can partially repair uremia-induced sciatic nerve injury (Figure 1).

In nerve cells, Na⁺, K⁺-ATPase coupling ATP hydrolysis (ATPase) is an energy conversion ion pump. It maintains Na⁺ and K⁺ gradients and participates in the release and absorption of neurotransmitters²⁵. ATP depletion leads to the decrease of axon ion concentration and axonal mutability, which is directly related to the excitability of neurons and the normal operation of axons²⁶. Meanwhile, succinate dehydrogenase (SDH) plays an important role in ATP production. The decrease of SDH reduces the production of ATP, induces the production of a large number of mitochondrial reactive oxygen species (ROS)²⁷. ROS induces oxidative stress and damage lipids, proteins, and DNA, leading to mitochondrial and cellular dysfunction, inflammation, and apoptosis^{28,29}. The content or activity of MDA, GSH-Px, and SOD determines the redox state of sciatic nerve³⁰. In our research, the injury of sciatic nerve reduces the activity of Na⁺, K⁺-ATPase, SDH, GSH-Px, SOD, and ATP production and induces the up-regulation of MDA content. Echinacoside and α -Klotho can significantly improve this situation (Figure 3). This suggested that Echinacoside and α -Klotho could improve uremia-induced sciatic nerve injury by altering the redox status of the sciatic nerve and inducing antioxidant protection.

Echinacoside is a natural compound with many biological functions, which was first discovered around 70 years ago. Liu J et al. reviewed the Echinacoside extraction process, pharmacological properties, and its potential mechanism in 2018¹². Echinacoside showed significant neuroprotective activity in animal and cell models of Parkinson's disease and Alzheimer's disease. It has several possible molecular

mechanisms, including mitogen-activated protein kinase (MAPK), NF- κ B, and reactive oxygen species (ROS)³¹⁻³³. NLRP3 is a spotted, damage-related adaptor protein molecule, which contains a caspase ASC. Its active molecule is pro-caspase-1³⁴. After ROS activates NLRP3 inflammasome, pro-caspase-1 is activated into mature Caspase-1. Cytokines IL-1 and IL-18 were activated through hydrolysis, leading to a rapid, pro-inflammatory form of cell death known as pyroptosis³⁵. It is characterized by cell swelling, cell membrane rupture, membrane pore formation, massive leakage of cytoplasmic contents, and high inflammation. The classical caspase-1-dependent pyroptosis pathways mainly include assembly of inflammatory corpuscles, activation of caspase-1 precursors, and cleavage of effector molecular GSDMD³⁶. NF- κ B is a transcription factor responding to extracellular signals. It is then transported to the nucleus to up-regulate the transcriptional of specific genes³⁷. Continuous activation of NF- κ B signals promotes the level of inflammatory mediators. There have been reports showing that this pattern of inflammatory cellular death plays an important role in progression of Parkinson³⁸. According to our current research, IL-1 β , IL-18, caspase-1, NLRP3, GSDMD, and NF- κ B (nucleus) expression were up-regulated in rats with sciatic nerve injury, suggesting that uremic induced sciatic nerve injury may be achieved by inducing pyroptosis of nerve cells. Echinacoside and α -Klotho can reverse this situation (Figure 2, Figure 4, Figure 5). α -Klotho proteins play an important role in neuronal activity and are key to aging and neurodegenerative processes³⁹. The current results also suggest that α -Klotho proteins are expressed in the injured sciatic nerve, while Echinacoside processing can improve the transcriptional and translational levels of α -Klotho (Figure 4 and Figure 5).

In conclusion, Echinacoside has a significant mitigation effect on uremia-induced sciatic nerve injury in rats. Its specific molecular mechanism is associated with inhibition of the classical cellular pyroptosis pathway, which is likely achieved by promoting α -Klotho expression. Further studies are needed to confirm our findings.

Authors' contributions

YZ and YY conceived and designed the study and drafted the manuscript. YZ, BG, HW, JM and ZG collected, analyzed and interpreted the experimental data. YY, JM and ZG revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

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